Volume 24 Issue 13
Originally issued as G1

Global Journal of Medical Research: G
Veterinary Science and Veterinary Medicine
Volume 24 Issue 1 Version 1.0 Year 2024
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals
Online ISSN: 2249-4618 & Print ISSN: 0975-5888

Effect of the Intravaginal Inoculation of Phytobiotics in a Murine Experimental Model

By Antonella Marchesi, Jessica Alejandra Silva, Priscilla Romina De Gregorio & María Elena Fátima Nader-Macías

Abstract- Lactobacilli play a fundamental role in maintaining health and preventing infections of the female urogenital tract. Complementary use of vegetal extracts selected for their ethnopharmacological characteristics and conventional uses for the different conditions in the human body as an adequate alternative therapy for the restoration of the vaginal microbiome has emerged. Compatibility of phytoextracts with lactobacilli for the design of phytobiotic formulas was determined previously. Safety of selected combinations by the intravaginal (i.va.) administration in a murine model was evaluated to determine if some type of adverse effect was produced in the host. Lactobacillus gasseri CRL1320, Limosilactobacillus reuteri CRL1324, Ligilactobacillus salivarius CRL1328 and Lacticaseibacillus rhamnosus CRL1332 combined with Hamamelis-virginiana, Amaranthus-muricatus and Smilax-áspera, were inoculated individually or combined in mice vagina (7 daily doses).

Keywords: *beneficial lactobacilli, vegetal-extracts, phytobiotics, safety, vaginal murine, adverse effect.*

GJMR-G Classification: *LCC: QP251*



Strictly as per the compliance and regulations of:

© 2024. Antonella Marchesi, Jessica Alejandra Silva, Priscilla Romina De Gregorio & María Elena Fátima Nader-Macías. This research/review article is distributed under the terms of the Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0). You must give appropriate credit to authors and reference this article if parts of the article are reproduced in any manner. Applicable licensing terms are at https://creativecommons.org/licenses/by-nc-nd/4.0/.

Effect of the Intravaginal Inoculation of Phytobiotics in a Murine Experimental Model

Antonella Marchesi [α], Jessica Alejandra Silva [σ], Priscilla Romina De Gregorio [ρ] & María Elena Fátima Nader-Macías [ω]

Abstract- Lactobacilli play a fundamental role in maintaining health and preventing infections of the female urogenital tract. Complementary use of vegetal extracts selected for their ethnopharmacological characteristics and conventional uses for the different conditions in the human body as an adequate alternative therapy for the restoration of the vaginal microbiome has emerged. Compatibility of phytoextracts with lactobacilli for the design of phytobiotic formulas was determined previously. Safety of selected combinations by the intravaginal (i.va.) administration in a murine model was evaluated to determine if some type of adverse effect was produced in the host. *Lactobacillus gasseri* CRL1320, *Limosilactobacillus reuteri* CRL1324, *Ligilactobacillus salivarius* CRL1328 and *Lacticaseibacillus rhamnosus* CRL1332 combined with *Hamamelis-virginiana*, *Amaranthus-muricatus* and *Smilax-áspera*, were inoculated individually or combined in mice vagina (7 daily doses). Vaginal washes were taken for microbiological (cultivable lactobacilli) and cytological (May Grünwald-Giemsa technique) evaluations, and vagina for histological (by Hematoxylin-Eosin) and ultrastructural (by electronic microscopy) analyses. Results obtained demonstrated that the i.va. phytobiotics administration did not produce adverse effects in the murine vaginal tract, by absence of inflammatory response. There were no modifications of the vaginal tract at the structural and ultrastructure level, suggesting the safety of phytobiotic formulas. Results obtained in this stage are original because information of viability and safety of natural extracts with strains of beneficial lactobacilli in *in vivo* trials are limited, and will allow progress in the design of beneficial formulas of reproductive age women.

Keywords: beneficial lactobacilli, vegetal-extracts, phytobiotics, safety, vaginal murine, adverse effect.

I. Introduction

Lactobacilli play a fundamental role in the urogenital tract by maintaining health or preventing infections through different mechanisms of action, demonstrated by a wide diversity of publications (Lazarenko et al., 2012; Karlsson et al., 2012; Wagner and Johnson et al., 2012; De Gregorio et al., 2014, 2015, 2016, 2019; Nader and Juárez-Tomas 2015; Nader Macías et al., 2021; Mashatan et al., 2023; Szczerbiec et al., 2024; Gupta et al., 2024). Plants extracts are applied to treat different pathologies in human and animals, which is one of the reasons to support their selection, supported by their ethnopharmacological characteristics to be used in urogenital tract infections (UGTI) prevention or treatment (Argentine Pharmacopoeia; Palmeira-de-Oliveira et al., 2015; Flower et al., 2016; Montorsi et al., 2016; Aziz y col., 2017: Moreno et al., 2018; Marchesi et al., 2020). Different products were applied for UGTI treatment, most of them derived from natural products, given the requirement of new therapies to prevent and treat chronic infections (Palmeira-de-Oliveira et al., 2013). In recent years, the frequent use of antimicrobials (such as antibiotics, antimicotics, and antivirals) is constantly questioned due to the appearance of resistant strains (Falagas et al., 2006; Flores-Mireles et al., 2015; Karam et al., 2019) which support the search for alternatives therapies or strategies to prevent or treat female urogenital infections, and to restore the microbiota of vaginal tract.

"*Probiotics*" are defined as "*live microorganisms, when administered in adequate amounts, evidence a beneficial physiological effect on the consumer*" (Hill et al., 2014). "*Pharmabiotics*" are "*living or dead microorganisms and their microbial constituents and metabolites that can beneficially interact with the host*" (Shanahan et al., 2009). "*Phytobiotic*" formulas refer to the "*combination of plant extracts with probiotic microorganisms to maintain or prevent health*" (Nader-Macías and Juárez-Tomás et al., 2015). "*Phytoextracts*" or "*Vegetal extracts*" are the substances obtained by maceration process of plants in 40% alcohol for medicinal use, approved by pharmacopoeias (Argentine Pharmacopoeia Method). Despite the fact that lactobacilli are generally recognized as safe (Generally Regarded as Safe, GRAS) by international organizations (FAO/WHO, 2012), and plant extracts which different applications in humans are described in pharmacopoeias (Argentine and European Pharmacopoeias) there is an imperative requirement to determine their safety and innocuity. It is of high importance to demonstrate that there is not production of adverse effects in animal models, before advancing in the evaluation of i.va. formulations in human clinical trials (Falagas et al., 2007; Nader-Macías et al., 2008;

Author α σ: Antonella Marchesi and Jessica Alejandra Silva have contributed at the same level in performing the experimental protocols.
Author ρ: Centro de Referencia para Lactobacilos-Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina (CERELA-CONICET). Chacabuco 145. San Miguel de Tucumán. 4000. Argentina.
Author ω: Prof. Dra. María Elena Fátima Nader-Macías CERELA-CONICET Chacabuco 145. 4000 San Miguel de Tucumán. Argentina. e-mails: fnader@cerela.org.ar, fatynader@gmail.com

© 2024 Global Journals

2021; Alfaro et al., 2013; Silva et al., 2023). Selection of strains and extracts was also performed to further design phytobiotic formulas aimed to treat, prevent or maintain the human vaginal health (Mishra et al., 2018).

Safety of vaginal lactobacilli was determined previously, supported by requirements established for the design of probiotic formulas for human beings. Intraurethral and i.va. administration of vaginal lactobacilli was applied in a murine experimental model evidencing their persistence in the urogenital tract, absence of adverse effects, protection against *Staphylococcus aureus*, *Streptococcus agalactiae*, *Escherichia coli* and *Candida albicans* challenges, and immune system modulation (Silva-Ruiz et al., 2004; Zárate et al., 2007; De Gregorio et al., 2012; 2015; 2016; 2019; Leccese-Terraf et al., 2015). Experimental animal model in BALB/c mice was used for its small size, easy manipulation and short reproductive cycle. Safety of lactobacilli was also demonstrated in phase I trial in healthy women (De Gregorio et al., 2020). Our research group demonstrated that the i.va. administration of functional nanofibers was safe in murine models, producing a viable lactobacilli increase and promoting their permanence in the murine vaginal tract (Silva et al., 2023).

The physiological characteristics of the murine vaginal tract are different from those of women, mainly in the neutral vaginal pH, the low numbers of lactobacilli in the autochthonous microbiota and the length and characteristics of the sexual cycle (Patras et al., 2013). McLean et al. (2012) described a simple and non-invasive protocol to determine the estrous cycle stage of female mouse without altering its reproductive cycle, similar to the one used in this work. Murine models success described by numerous scientists to evaluate the vaginal tract is useful to predict the expected behaviour in human beings (Silva-Ruiz et al., 2001; Zárate et al., 2009; Muench et al., 2009; Spurbeck and Arvidson 2011; Joo et al., 2011; Joo et al., 2012; Patras et al., 2013; De Gregorio et al., 2014; 2015; 2016; 2019).

Phytobiotics were formulated previously in our research group with 30 different beneficial vaginal lactobacilli (BVL) strains combined with phytoextracts (selected by their application and characteristics related with vaginal health improvement) to produce a synergic or complementary pharmacological effect (Marchesi et al., 2020). However, no safety assays were published referred to formulas designed with vegetal extracts and probiotic bacteria combined, administered by the vaginal way. Thus, the aim of this work was to evaluate the safety of phytobiotic formulas designed with beneficial lactobacilli and vegetal extracts combined, in order to define the permanence of the strains, and if some type of adverse reactions was evidenced in the murine experimental model.

II. Results and Discussion

a) Quantification of Viable Lactobacilli in Vaginal Washing

The results obtained when evaluating the number of viable lactobacilli from vaginal washing (v.w.) of mice inoculated with the different phytobiotics did not show significant differences ($p>0.05$) referred to mice inoculated only with BVL (control), indicating that a stimulatory or inhibitory effect by the phytoextracts on the colonization capability of lactobacilli was not produced (Fig.1). The comparison of the i.va. inoculation of the four BVL strains, showed higher viable cell numbers of *L. gasseri* CRL1320 (10^6 CFU/ml v.w.), while *L. reuteri* CRL1324 and *L. salivarius* CRL1328 were in a lower value (10^{2-3} CFU/ml). In control mice, viable lactobacilli were not detected (in MRS-pH-5.5, selective medium) suggesting that the vaginal isolated lactobacilli came from the exogenous administration. Mice receiving phytobiotics-*L. rhamnosus* CRL1332showed similar values than *L. gasseri* CRL1320. *L. salivarius* CRL1328+phytocompounds mice produced a higher number of viable lactobacilli, but not significant compared with the strain without vegetal extracts. At the end, *L. salivarius* and *L. rhamnosus* demonstrated a higher colonization in mouse vagina when administered in phytobiotic formulas. These results were different to those obtained from *in vitro* assays (Marchesi et al., 2020) where some vegetal-extracts showed a stimulatory or inhibitory effect on BVL. Differences between *in vitro* and *in vivo* assays were also observed in BVL interaction with *St. agalactiae* (De Gregorio et al., 2014). These differences support the requirement to apply different criteria for probiotic selection, including both *in vitro* an *in vivo* safety assays (Nader-Macias and Juárez, 2015; Nader-Macias et al., 2021). Viable lactobacilli quantification in v.w. were similar to those obtained previously when the optimal i.va. dose of *L. reuteri* CRL1324 was determined (De Gregorio et al., 2015).

© 2024 Global Journals

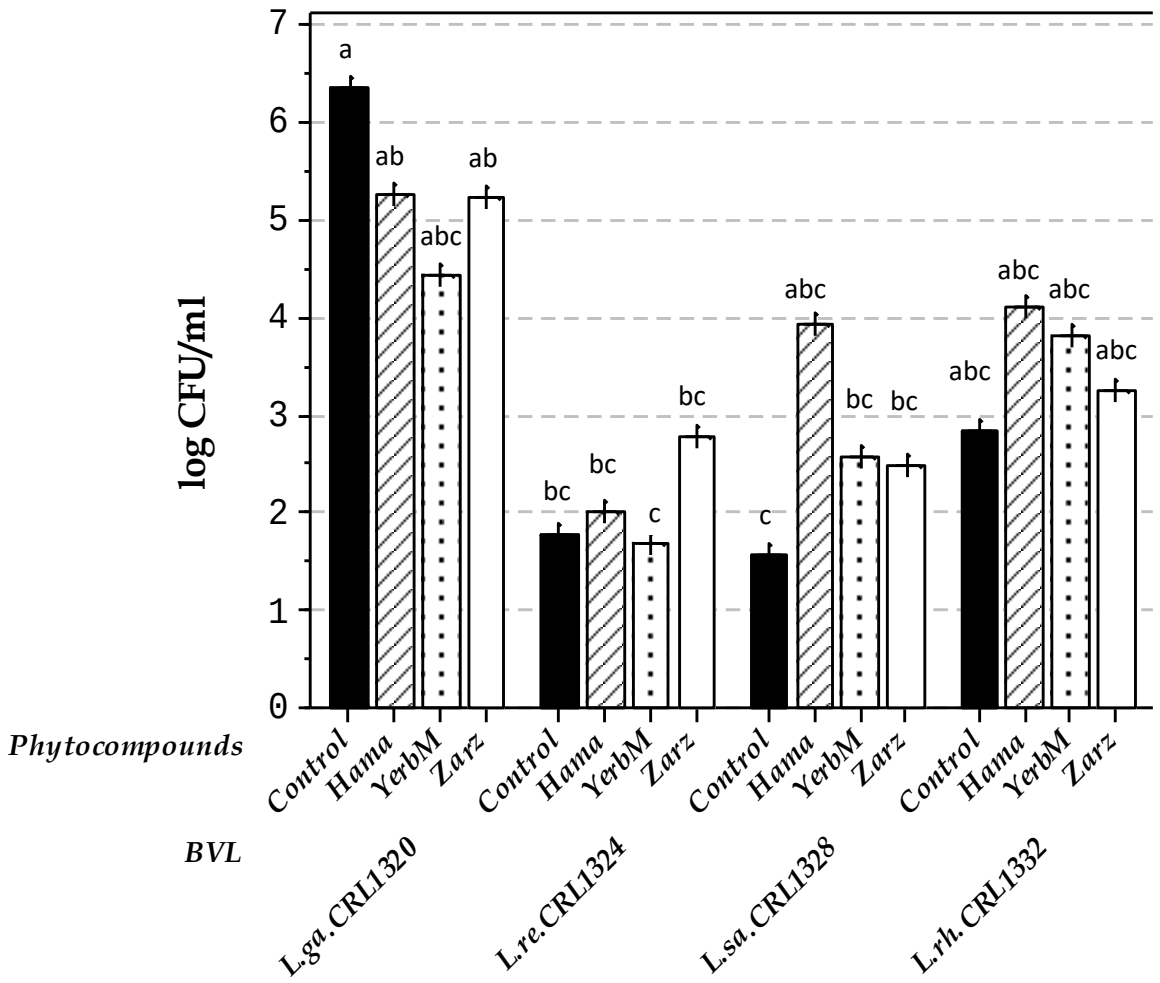


Fig.1: Quantification of viable BVL in murine v.w. of mice inoculated with lactobacillivegetal extracts: Control (black-bar: ■), *Hama:Hamamelis* (oblique-bar: ▨), *YerbM:Yerba meona* (dot-bar: ⬚) and *Zarz*: *Zarzaparilla* (white-bar: □). The results represent the log CFU/ml mean values of *L. gasseri* CRL1320, *L. reuteri* CRL1324, *L. salivarius* CRL1328, and *L. rhamnosus* CRL1332 ± standard error. Significant differences in the number of each BVL strain and their combination with extracts are indicated by different letters ($p < 0.05$).

b) Vaginal Cytology

Intravaginal administration of phytobiotic formulas did not produce adverse effect or inflammatory response in the murine experimental model. May Grünwald-Giemsa vaginal smears from phytobiotics, BVL or vegetal extracts inoculated mice obtained, by optical microscopy are included in Fig.2.A. Cytological evaluation evidenced absence of adverse effect at this level. All samples showed similar patterns to control mice, indicating pseudo-estrous state induced by hormonal inoculation, characterized by the presence of keratinized epithelial cells (irregular shape of scales) and absence of nucleated epithelial cells and leukocytes (which are indicative of an inflammatory or adverse response). Safety of BVL in the urogenital tract was also demonstrated previously. Silva de Ruiz et al. (2003) administered intraurethrally *L. fermentum* CRL1508 in a murine model with no adverse effect or significant changes in the organs (kidney, ureter, bladder or urethra) at the structural and ultra-structural level, indicating its safety. De Gregorio et al. (2012) evidenced that the administration of five different human BVL to BALB/c-mice for 4 163 days did not produce adverse effects in cytological and immunological assays. Zarate et 164 al. (2009) also showed the protection against *S. aureus* challenge by the i.va. 165 administration of human BVL strains with no adverse effects. Recently, Silva et al. (2023) demonstrated the absence inflammatory response at the cytological level after the i.va. administration of nanofibers with *L. rhamnosus* CRL1332 immobilized in murine model.

© 2024 Global Journals

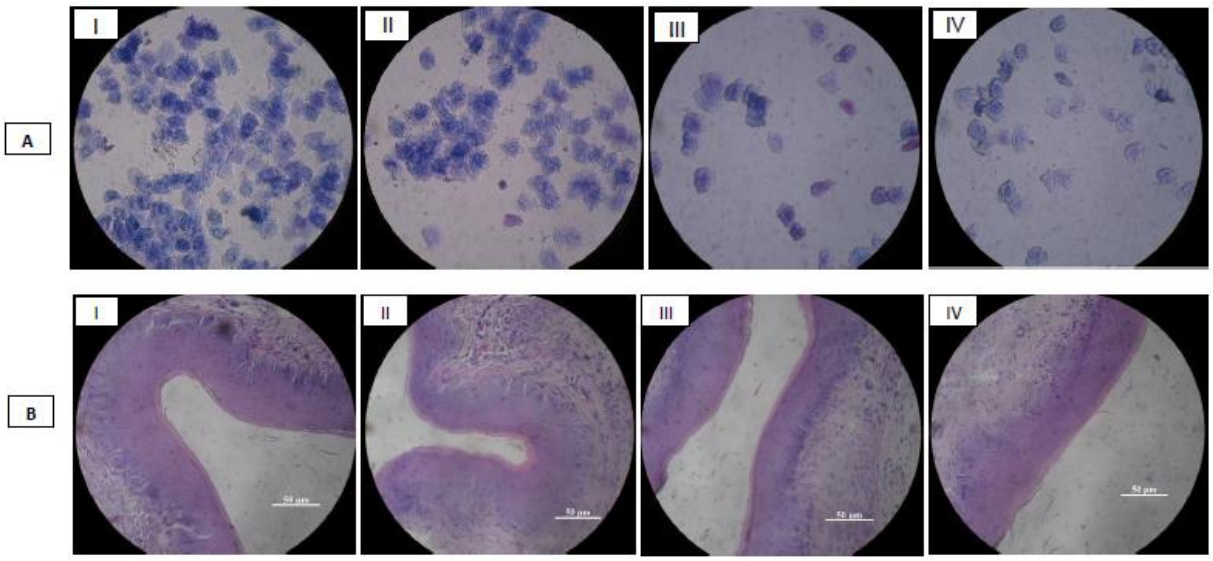


Fig. 2: Vaginal Cytology (A) and Histology (B) images of BALB/c mice after 171 intravaginal administration with different phytobiotics.A-I: *L. rhamnosus* 172 CRL1332+*Hamamelis;*A-II: *Hamamelis;* A-III: *L. reuteri* CRL1324+*Hamamelis;*A173 IV: Control. B-I: *L. gasseri* CRL1320+*Hamamelis*; B-II: *Hamamelis*; B-III: *L. gasseri* 174 CRL1320; B-IV: Control.

c) Vaginal Histology

Microscopy evaluation is useful to facilitate diagnosis, as some authors claim. Donders 178 et al. (2019) recommend the use of microscopy for an exact observation of vaginal 179 epithelium condition, and helps to define different therapeutic alternatives. Histological 180 structure of vaginal tract of mice i.va. inoculated with phytobiotics, phytocompounds or 181 lactobacilli showed normal lamina propia characteristics, multilayer epithelium, 182 keratinized epithelial cells, indicating the pseudo-estrous state (Fig. 2. B). The observed 183 pattern was similar to control mice (Fig. 2. B-IV), with absence of inflammatory response 184 in murine tissue.

Zarzaparilla has antifungal, antiseptic and diuretic uses; *Hamamelis* has antiviral, antiseptic and anti-inflammatory activities, while *Yerba meona* evidenced diuretic, antitumor, drastic purging, warts, herpes, depurative, and other applications, supporting their selections to evaluate the beneficial properties when used in combination with BVL (Argentine-Pharmacopoeia; European-Pharmacopoeia; Theisen et al., 2014; Qi et al., 2017; Marchesi et al., 2020). Different scientists published the effect of plant derivatives administered orally or intravaginally in women suggesting a variety of effects and mechanisms of action. Moraes et al. (2012) demonstrated the efficacy and safety of *Mentha crispa* as a suitable alternative in the therapy of *Trichomonas vaginalis* infections. Satthakarn et al. (2015) administered *Houttuynia cordata* aqueous-extract in women urogenital tract determining an increase of cells participating in vaginal immune response. Espino et al. (2019) showed the complementary antifungal effect when administering *L. plantarum* cream+two extracts in candidiasis treatment in *in vitro* protocols. No publications were detected referred to protocols of i.va. administration of phytobiotics in a murine experimental models. Recently, Miranda and Nader-Macías (2024) showed that the i.va. administration of probiotic and phytobiotic formulations to pregnant female cows at pre and postpartum increased significantly the number of lactic acid bacteria with no adverse local and systemic effects in cows.

d) Ultrastructure of Murine Vagina

Ultrastructure of murine vagina inoculated with phytobiotics was characterized by a keratinized epithelium with anucleated polystratified cells, supported on the lamina propia with anucleated cells. Microphotographs of the vaginal tract of lactobacilli and phytocompounds i.va. inoculated mice did not evidence modifications, being similar in experimental group (*L. salivarius* CRL1328+*Hamamelis)* (Fig.3.A,B) and control mice (Fig. 3. G,H). Absence of inflammatory response, ultrastructure maintenance, and no other cells participating in inflammation indicate no adverse effect. Normal polystratified murine vaginal epithelium was evidenced. Mice inoculated with phytobiotics and lactobacilli showed bacteria or bacilli close or in contact with the epithelial cells surface (black arrows). These bacteria were not detected in mice i.va. inoculated only with phytoextracts or control animals. Therefore, it could be suggested that the bacteria evidenced in vagina of mice are those i.va. inoculated for 7-days, with no adverse effects. As control mice did not show bacteria, lactobacilli permanence is supported by their exogenous administration to mice.

© 2024 Global Journals

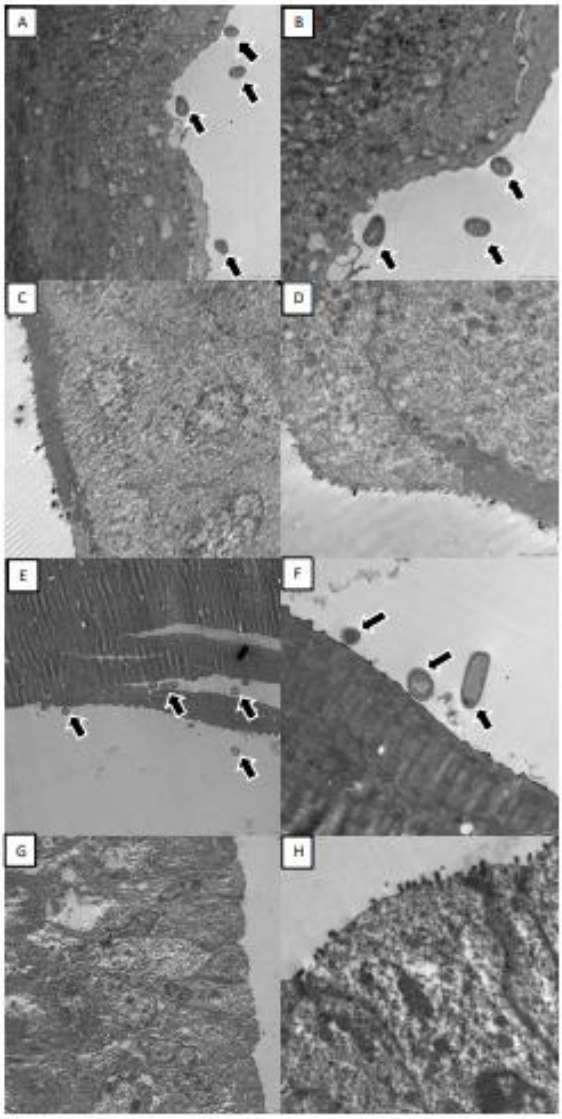


Fig. 3: Ultrastructure of mouse vagina i.va. inoculated with phytobiotics, lactobacilli or phytocompounds analysed by transmission electron microscopy. A:*L. salivarius* CRL1328+*Hamamelis* (2500X); **B:** *L. salivarius* CRL1328+*Hamamelis* (4000X); **C:** *Hamamelis* (1200X); **D:** *Hamamelis* (4000X); **E:** *L. salivarius* CRL1328 (1200X); **F:** *L. salivarius* CRL1328 (4000X); **G:** Peptone-water Control Mice Group (800X); **H:** peptone-water Control Mice Group (4000X). Bacteria or bacilli close or in contact with the epithelial vaginal cells are indicated with black arrows.

III. Materials and Methods

Microorganisms: Four BVL strains previously characterized and genetically identified were used in this work:*Lactobacillus gasseri* CRL1320, *L. reuteri* CRL1324, *L. salivarius* CRL1328 and *L. rhamnosus* CRL1332 (Marchesi et al., 2020) (Table 1). The strains were freezed-stored in milk yeast-extract (10% skim-milk, 0.5% yeast-extract, 1% glucose), and they were inoculated, and subcultured 3 times in MRS broth (De-ManRogosa and Sharpe) (Biokar-Diagnostics-Beauvais-France) at 37°C for 13-14 h before their use. For the i.va. administration in mice, the strains were centrifuged at 3000g for 10 min (Presvac-Argentina), and resuspended in 50μl agarose-peptone [1% meatpeptone, 1.5% agar] or combined with the vegetal extracts selected (Table 2).

© 2024 Global Journals

Table 1: Beneficial Vaginal Lactobacilli (BVL) Properties

BVL Strains	Beneficial Properties
L. gasseri CRL1320	H_2O_2 and lactic acid production, high hydrophobicity, pathogens inhibition, biofilm formation
L. reuteri CRL1324	H_2O_2 and lactic acid production, high hydrophobicity, biofilm formation, pathogens inhibition, colonization, of BALB/c vaginal tract mice, adhesion to fibrinogen and mucin
L. salivarius CRL1328	bacteriocin production, pathogens inhibition
L. rhamnosus CRL1332	H_2O_2 production, high hydrophobicity, biofilm, formation, high resistance to lyophilization, pathogens inhibition, colonization of BALB/c mice vaginal tract, adhesion to fibrinogen and mucin

Table 2: Vegetal Extracts and uses

Scientific name	Popular name	Uses	Pharmacopoeia
Hamamelis viriginiana	*Hamamelis*	Astringent, antiseptic, antiinflammatory, antiviral, venotonic	Argentine 8th edition
Amaranthus muricatus	*Yerba-meona*	Diuretic, antitumor, drastic purging, warts, herpes, depurative	Argentine 6th edition
Smilax aspera	*Zarzaparrilla*	Diuretic renal depurative, antibacterial, antifungal, antiseptic	European

Phytocompounds: "*Hamamelis-virginiana*" (*Hamamelis),* "*Amaranthus-muricatus*" *(Yerba meona)* and "*Smilax-áspera" (Zarzaparilla)* were selected for their ethnopharmacological properties and compatibility against different BVL, summarized in Table 2 (Marchesi et al., 2020). From dry extracts of *Hamamelis* (leaves), *Yerbameona* (leaves-stem), and *Zarzaparilla* (bark+branches+fruits), previously obtained by maceration (according to the Argentine Pharmacopoeia) and dried, 1 mg of extract was weighed with 1 ml of alcohol 40%, taking only 20 *µ*l of each plant extract (1mg/ml in 40%-alcohol) were mixed with 30*µ*l agarose-peptone, 50*µ*l i.va. administered to mice.

Microorganisms+Phytocompounds: the pellet from 3rd BVL subculture was mixed with 20µl vegetal-extract+30µl agarose-peptone for mouse i.va. administration. The protocol of administration in the murine model is indicated in Figure 4.

© 2024 Global Journals

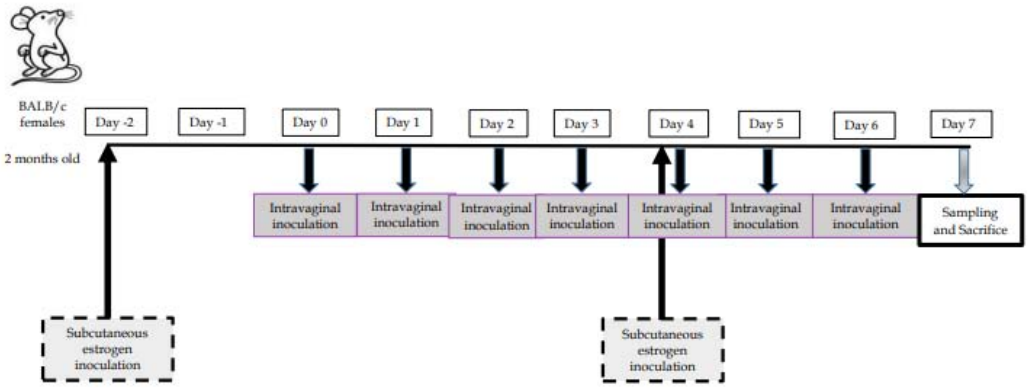


Fig. 4: Protocol of Administration of Phytobiotics, Lactobacilli and Phytocompounds by I.Va. Route to Estrogenized-Adult BALB/C Female Mice

a) Female BALB/C Mice as Experimental Model

Female BALB/c mice, 45-days-old, 20-25 average-weight, were provided and maintained in CERELA nursery, keeping constant environmental conditions, fed *ad libitum* with conventional balanced diet. Pseudostrous status was induced with β-estradiol-valerate subcutaneous administered on days "-2" and "3", according to previously set-up protocols, to avoid the variations from the estrous cycle state, and to promote lactobacilli permanence (De Gregorio et al., 2012). Hormone was prepared from a stock solution (2 mg/ml, Sigma-Life-Sciences, Switzerland) resuspended and diluted in sesame-oil (Sigma-Life-Sciences, Mexico) at 0.2 mg/ml. Then, 0.1 ml was injected subcutaneously (0.02 mg). The experimental protocol, hormone administration and sampling days are shown in Figure 4. 100 mice were used, divided in 20 groups, assigning 5 mice randomly to each group, as follows:

1) *Phytobiotic Groups (12):* The following combinations of probiotic BVL+phyto extracts were i.va administered for 7 days: *L. gasseri* CRL1320+ *Hamamelis*, *L. gasseri* CRL1320+ *Zarzaparrilla*, *L. gasseri* CRL1320+*Yerba-meona*, *L. reuteri* CRL1324+*Hamamelis,L. reuteri* CRL1324+ *Zarzaparrilla*, *L. reuteri* CRL1324+ *Yerbameona*, *L. salivarius* CRL1328+*Hamamelis, L. salivarius* CRL1328+*Zarzaparrilla, L. salivarius* CRL1328+ *Yerba-meona*, *L. rhamnosus* CRL1332+ *Hamamelis, L. rhamnosus* CRL1332+*Zarzaparrilla,* and *L. rhamnosus* CRL1332+*Yerba-meona*.
2) *Vegetal Extracts Groups (3):* mice were i.va. administered for 7 days with: *Hamamelis-virginiana* (*Hamamelis*), *Amaranthus-muricatus* (*Yerba-meona*) and *Smilaxáspera* (*Zarzaparrilla*).
3) *BVL Strains Groups (4):Lactobacillus gasseri* CRL1320, *Limosilactobacillus reuteri* CRL1324, *Ligilactobacillus salivarius* CRL1328 or *Lacticaseibacillus rhamnosus* CRL1332 were individually i.va. administered during 7 days, at 10^7-10^8 CFU each dose:
4) *Control Group (1):* 20µl saline+30µl 1%-agarose-peptone.

Two independent assays were carried out. CERELA Institutional Committee for the Care and Use of Laboratory Animals approved the experimental protocol CRL-BIOT-LMP2010/1A.

b) Mice Sampling and Analytical Procedures

Murine vagina was washed in sterile conditions with 50µl phosphate-buffered-saline (PBS: 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4, 140mM NaCl, pH 7.2) 7 times and v.w. of each animal pooled. Vaginal washes were used for different assays:

1) *Quantification of microorganisms:* serial dilutions were prepared and inoculated in selective MRS agar (pH 5.5) to quantify viable BVL after 48 h incubation at 37°C. Number of microorganisms was expressed as log CFU/ml v.w.
2) *Cytological studies:* 10µl aliquots were spread on slides, fixed and stained with MayGrünwald-Giemsa technique to assess whether inoculation with phytobiotics produced any type of adverse effect at cytological level. Preparations were observed under light microscope (40x, Axio-Scope-A1, Carl-Zeiss) (McLean et al., 2012).
3) *Histological studies:* mice were sacrificed by cervical dislocation at the 7th day and dissected to extract vagina, which was transferred to the appropriate solvents for subsequent process for histological and electronic microscope observation, as follows:
   i. *Histological evaluation (light microscopy):* vaginal tissues were fixed in 4% (v/v) formaldehyde at 4°C, embedded in paraffin by applying routine laboratory methods. Organs were processed according to Silva de Ruiz et al. (2003) using Carl Zeiss Microscope (40x).
   ii. *Ultrastructural evaluation (transmission electron microscopy):* mouse vagina samples were placed in Karnovsky's fixative (2.66% paraformaldehyde,

© 2024 Global Journals

0.1M sodiumphosphate-buffer, pH 7.4, 1.66% glutaraldehyde) for 1 week. The technique applied was detailed previously (Zampini et al., 2020). Samples were processed and observed at the Zeiss Libra 120 electron microscope (Carl Zeiss, Oberkochen, Germany) of Integral Center for Electronic Microscopy in Tucumán (CIME-CONICET).

c) *Statistics Analysis*

Analysis of variance (ANOVA) using a general linear model was applied to define the main effects of experimental groups on the number of viable lactobacilli. Significant differences (p-value$<$0.05) between mean values were determined by Tukey's test, using MINITAB statistical software (version-16 for Windows).

Acknowledgements

This work was supported by CONICET and ANPCYT grants.

References Références Referencias

1. Alfaro, M., Manrique-Rodríguez, S., and Fernández-Llamazares, C.M. (2013). Empleo clínico de los probióticos y aspectos prácticos de su empleo. Nutr.Hosp.Madrid. Vol.28, supl.1.
2. Argentina, F. 6°-7°-8° and 9°ed. Ministerio-de-Salud-de-la-Nación-ANMAT. (2018). Farmacopea Argentina.
3. Aziz, M. A., Khan, A. H., Adnan, M., & Izatullah, I. (2017). Traditional uses of medicinal plants reported by the indigenous communities and local herbal practitioners of Bajaur Agency, Federally Administrated Tribal Areas, Pakistan. *Journal of Ethnopharmacology*, 198, 268–281.
4. Daniele, M., Pascual, L., and Barberis, L. (2014). Curative effect of the probiotic strain *Lactobacillus fermentum* L23 in a murine model of vaginal infection by *Gardnerella vaginalis*. *Lett.Appl .Microbiol.* 59, 93-98.
5. De Gregorio, P.R., Juárez-Tomás, M.S., Santos, V., and Nader-Macías, M.E.F. (2012). Beneficial lactobacilli: effects on the vaginal tract in a murine experimental model. *Antonie van Leeuwenhoek.* 102, 569-580.
6. De Gregorio, P.R., Juarez-Tomás, M.S., Leccese-Terraf, M.C., and Nader-Macías, M.E.F. (2014). *In-vitro* and *in-vivo* effects of beneficial vaginal lactobacilli on pathogens responsible for urogenital tract infections.*J.Med.Microbiol.* 63, 685696.
7. De Gregorio, P.R., Juárez-Tomás, M.S., Leccese-Terraf, M.C., and Nader_Macías, M.E.F. (2015). Preventive effect of *Lactobacillus reuteri* CRL1324 on group-B *Streptococcus* vaginal colonization in an experimental mouse model. *J.Appl.Microbiol.* 118, 1034-1047.
8. De Gregorio, P.R., Juárez-Tomás, M.S., and Nader_Macías, M.E.F. (2016). Immunomodulation of *Lactobacillus reuteri* CRL1324 on group-B *Streptococcus* vaginal colonization in a murine experimental model. *Am.J. Reprod. Immunol.* 75, 23-35.
9. De Gregorio, P.R., Maldonado, N.C., Pingitore, E.V., Leccese-Terraf, M.C., JuarezTomás, M.S., Silvia-de-Ruiz, C.S., Santos, V., Wiese, B., Bru, E., Paiz M.C., Reina M.F.,Schujman, D.E., and Nader-Macías, M.E.F (2020). Intravaginal administration of gelatine capsules containing freeze-dried autochthonous lactobacilli: a double-blind, randomised clinical trial of safety. *Benef.Microbes.* 11, 5-17.
10. De Gregorio, P.R., Silva, J.A., Marchesi, A., and Nader-Macías, M.E.F. (2019). AntiCandida activity of beneficial vaginal lactobacilli in *in-vitro* assays and in a murine experimental model. *FEMS.Yeast.Res.* 19, foz008.
11. Donders, G.G.G., Ruban, K., Bellen, G., and Grinceviciene, S. (2019). Pharmacotherapy for the treatment of vaginal atrophy. *Expert.Opin.-Pharmacother.* 20, 821-835.
12. Espino, M., Solari, M., de-los-Ángeles Fernández, M., Boiteux, J., Gómez, M.R., and Silva, M.F. (2019). NADES-mediated folk plant extracts as novel antifungal agents against *Candida albicans*. *J.Pharm.Biomed.Anal* 167, 15-20.
13. European Farmacopea. European Directorate for the Quality of Medicines & Health-Care (EDQM), 2018.
14. Falagas, M. E., Betsi, G.I., and Athanasiou, S. (2007). Probiotics for the treatment of women with bacterial vaginosis. *Clin.Microbiol.Infect.* 13, 657-664.
15. Falagas, M.E., Betsi, G.I., Tokas, T., and Athanasiou, S. (2006). Probiotics for prevention of recurrent urinary tract infections in women. *Drugs.* 66, 1253-1261.
16. FAO/WHO Report. Evaluation of health and nutritional properties of powder milk and live lactic acid bacteria. (2012) http://www.fao.org/es/ESN/Probio/probio.htm Flores-Mireles, A.L., Walker, J.N., Caparon, M., and Hultgren, S.J. (2015). Urinary tract infections: epidemiology, mechanisms of infection and treatment options. *Nat.Rev.Microbiol.* 13, 269-284.
17. Flower, A., Harman, K., Lewith, G., Moore, M., Bishop, F. L., Stuart, B., & Lampert, N. (2016). Standardised Chinese herbal treatment delivered by GPs compared with individualised treatment administered by practitioners of Chinese herbal medicine for women with recurrent urinary tract infections (RUTI): study protocol for a randomised controlled trial. *Trials*, 17, 358.
18. Gupta, V., Mastromarino, P., & Garg, R. (2024). Effectiveness of Prophylactic Oral and/or Vaginal

© 2024 Global Journals

Probiotic Supplementation in the Prevention of Recurrent Urinary Tract Infections: A Randomized, Double-Blind, Placebo-Controlled Trial. *Clinical infectious diseases: an official publication of the Infectious Diseases Society of America*, 78(5), 1154–1161.

19. Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S., Calder, P.C., and Sanders, M.E. (2014). The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat.Rev. Gastroenterol. Hepatol.* 11, 506-514.
20. Joo, H.M., Hyun, Y.J., Myoung, K.S., Ahn, Y.T., Lee, J.H., Huh, C.S., Han, M.J., and Kim, D.H. (2011). *Lactobacillus johnsonii* HY7042 ameliorates *Gardnerella vaginalis*-induced vaginosis by killing *Gardnerella vaginalis* and inhibiting NFκB activation. *Int.Immunopharmacol.* 11, 1758–1765.
21. Joo, H.M., Kim, K.A., Myoung, K.S., Ahn, Y.T., Lee, J.H., Huh, C.S., Han, M.J., & Kim, D.H. (2012). *Lactobacillus helveticus*HY7801 ameliorates vulvovaginal candidiasis in mice by inhibiting fungal growth and NF-κB activation. *Int.Immunopharmacol.* 14, 39–46.
22. Karam, M.R.A., Habibi, M. and Bouzari, S. (2019). Urinary tract infection: Pathogenicity, antibiotic resistance and development of effective vaccines against Uropathogenic *Escherichia coli*. *Mol.Immunol.* 108, 56-67.
23. Karlsson, M., Scherbak, N., Reid, G., and Jass, J. (2012). *Lactobacillus rhamnosus* GR1 enhances NF-kappaB activation in *Escherichia coli*-stimulated urinary bladder cells through TLR4. *BMC.microbial.* 12, 15.
24. Lazarenko, L., Babenko, L., Sichel, L. S., Pidgorskyi, V., Mokrozub, V., Voronkova, O., and Spivak, M. (2012). Antagonistic action of lactobacilli and bifidobacteria in relation to *Staphylococcus aureus* and their influence on the immune response in cases of intravaginal staphylococcosis in mice. *Probiotics.Antimicrob.Proteins.* 4, 78-89.
25. Leccese-Terraf, M.C., Juárez-Tomás, M.S., Nader‐Macías, M.E.F., and Silva, C. (2012). Screening of biofilm formation by beneficial vaginal lactobacilli and influence of culture media components. *J.Appl.Microbiol.* 113, 1517-1529.
26. Leccese-Terraf, M.C., Tomás, M.S. J., Rault, L., Le Loir, Y., Even, S., and Nader-Macías, M.E.F. (2017). In-vitro effect of vaginal lactobacilli on the growth and adhesion abilities of uropathogenic *Escherichia coli*. *Arch.Microbiol.* 199, 767-774.
27. Marchesi, A., Silva, J., Ficoseco, C.A., Wiese, B., and Nader-Macias, M.E.F. (2020). Effect of phytoderivatives on the growth of homologous beneficial vaginal lactobacilli (BVL) strains and their compatibility for the design of phytobiotics for the vaginal tract health.*WJPPS* 9, 3.
28. Mashatan, N., Heidari, R., Altafi, M., Amini, A., Ommati, M. M., & Hashemzaei, M. (2023). Probiotics in vaginal health. *Pathogens and disease*, 81, ftad012.
29. McLean, A.C., Valenzuela, N., Fai, S., and Bennett, S.A. (2012). Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification. *J.Vis.Exp.* e4389.
30. Miranda, M. H., & Nader-Macías, M. E. F. (2024). Pharmabiotic/phytobiotic formulas approach and their intravaginal effect on different parameters. *Veterinary Research Communications*, 1-15.
31. Mishra, N.N., Kesharwani, A., Agarwal, A., Polachira, S.K., Nair, R., and Gupta, S.K. (2018). Herbal gel formulation developed for anti-human immunodeficiency virus (HIV)-1 activity also inhibits *in vitro* HSV-2 infection. Viruses. 10, 580.
32. Montorsi, F., Gandaglia, G., Salonia, A., Briganti, A., & Mirone, V. (2016). Effectiveness of a Combination of Cranberries, *Lactobacillus rhamnosus*, and Vitamin C for the Management of Recurrent Urinary Tract Infections in Women: Results of a Pilot Study. *European Urology*, 70(6), 912–915.
33. Moraes, M.E.A., Cunha, G.H., Bezerra, M.M., Fechine, F.V., Pontes, A.V., Andrade, W. S., Frota Bezerra, F.A., O Moraes, M., and Cavalcanti, P.P. (2012). Efficacy of the *Mentha-crispa* in the treatment of women with *Trichomonas-vaginalis* infection. *Arch.Gynecol.Obstet.* 286, 125-130.
34. Moreno, M. A., Gómez-Mascaraque, L. G., Arias, M., Zampini, I. C., Sayago, J. E., Ramos, L. L. P., Schmeda-Hirschmann, G., López-Rubio, A., & Isla, M. I. (2018).
35. Electrosprayed chitosan microcapsules as delivery vehicles for vaginal phytoformulations. *Carbohydrate polymers*, 201, 425–437.
36. Muench, D.F., Kuch, D.J., Wu, H., Begum, A.A., Veit, S.J., Pelletier, M., Soler‐García, Á.A., and Jerse, A.E. (2009). Hydrogen Peroxide–Producing Lactobacilli Inhibit Gonococci *in-vitro* but Not during Experimental Genital Tract Infection. *J.Infect.Dis.* 199, 1369–1378
37. Nader-Macías, M. E. F., De Gregorio, P. R., & Silva, J. A. (2021). Probiotic lactobacilli in formulas and hygiene products for the health of the urogenital tract. *Pharmacology research & perspectives*, 9(5), e00787.
38. Nader-Macías, M.E.F., and Juárez-Tomás, M.S. (2015). Profiles and technological requirements of urogenital probiotics. *Adv.Drug.Deliv.Rev.* 92, 84-104.
39. Nader-Macías, M.E.F., Otero, M.C., Espeche, M.C., and Maldonado, N.C. (2008). Advances in the design of probiotic products for the prevention of

© 2024 Global Journals

major diseases in dairy cattle. *J.Ind. Microbiol. Biotechnol*. 35, 1387-1395.
40. Palmeira-de-Oliveira, A., Silva, B.M., Palmeira-de-Oliveira, R., Martinez-de-Oliveira, J., and Salgueiro, L. (2013). Are plant extracts a potential therapeutic approach for genital infections? *Curr.Med.Chem*. 20, 2914-2928.
41. Palmeira-de-Oliveira, R., Palmeira-de-Oliveira, A., & Martinez-de-Oliveira, J. (2015). New strategies for local treatment of vaginal infections. *Advanced drug delivery reviews*, 92, 105–122.
42. Patras, K.A., Wang, N.Y., Fletcher, E.M., Cavaco, C.K., Jimenez, A., Garg, M., Fierer, J., Sheen, T.R., Rajagopal, L., and Doran, K.S. (2013). Group-B *Streptococcus* CovR regulation modulates host immune signalling pathways to promote vaginal colonization. *Cell.Microbiol.* 15, 1154-1167.
43. Qi, Z.C., Shen, C., Han, Y.W., Shen, W., Yang, M., Liu, J.Zong-Suo, L., Pan, L., and Fu, C.X. (2017). Development of microsatellite loci in Mediterranean sarsaparilla (*Smilax-aspera; Smilacaceae*) using transcriptome data. *Appl.Plant.Sci.* 5, 1700005.
44. Satthakarn, S., Hladik, F., Promsong, A., and Nittayananta, W. (2015). Vaginal innate immune mediators are modulated by a water extract of *Houttuynia-cordata Thunb.BMC.Complement.Altern. Med.* 15, 1-8.
45. Silva-de-Ruiz, C., Del R. Rey, M., and Nader‐Macías, M.E. (2003). Structural and ultrastructural studies of the urinary tract of mice inoculated with *Lactobacillus fermentum.BJU.Int.* 91, 878-882.
46. Silva-de-Ruiz, C., Rey, M.D.R., Pesce-de-Ruiz Holgado, A., and Nader-Macías, M.E. (2001). Experimental administration of estradiol on the colonization of *Lactobacillus fermentum* and *Escherichia coli* in the urogenital tract of mice. *Biol.Pharm.Bull*. 24, 127–134.
47. Silva, J. A., De Gregorio, P. R., & Nader-Macías, M. E. F. (2023). Safety and Effects of
48. Intravaginal Administration of *Lacticaseibacillus rhamnosus* CRL1332 Immobilized on Nanofibers in a Murine Experimental Model. *Applied Microbiology*, 3(3), 1013-1026.
49. Spurbeck, R.R., and Arvidson, C.G. (2011). Lactobacilli at the front line of defense against vaginally acquired infections. *Future. Microbiol.* 6, 567-582.
50. Szczerbiec, D., Słaba, M., & Torzewska, A. (2023). Substances secreted by *Lactobacillus* spp. from the urinary tract microbiota play a protective role against Proteus mirabilis infections and their complications. *International Journal of Molecular Sciences*, 25(1), 103.
51. Theisen, L.L., Erdelmeier, C.A., Spoden, G.A., Boukhallouk, F., Sausy, A., Florin, L., and Muller, C.P. (2014). Tannins from *Hamamelis-virginiana* bark extract: characterization and improvement of the antiviral efficacy against influenza A virus and human papillomavirus. *PloS.one.* 9, e88062
52. Wagner, R.D., and Johnson, S.J. (2012). Probiotic *Lactobacillus* and estrogen effects on vaginal epithelial gene expression responses to *Candida albicans*. *J.Biomed.Sci.*19, 1-8.
53. Zampini, R., Castro-González, X. A., Sari, L. M., Martin, A., Diaz, A. V., Argañaraz, M. E., & Apichela, S. A. (2020). Effect of Cooling and Freezing on Llama (*Lama glama*) Sperm Ultrastructure. *Frontiers in veterinary science*, 7, 587596.
54. Zárate, G., Santos, V., and Nader-Macias, M.E. (2007). Protective effect of vaginal *Lactobacillus paracasei* CRL1289 against urogenital infection produced by *Staphylococcus aureus* in a mouse animal model. *Infect.Dis.Obstet.Gynecol.* 2009: 48358.
55. Zárate, G., Santos, V., and Nader-Macias, M.E. (2009). Protective effect of vaginal *Lactobacillus paracasei* CRL1289 against urogenital infection produced by *Staphylococcus aureus* in a mouse animal model. *Infect.Dis.Obstet.Gynecol.* 48358.

© 2024 Global Journals